APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1990, p. 1509–1515
0099-2240/90/061509-07$02.00/0

Vol. 56, No. 6

Isolation, Identification, and Characterization of a Feather-Degrading Bacterium†

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Received 16 November 1989/Accepted 2 March 1990

A feather-degrading culture was enriched with isolates from a poultry waste digestor and adapted to grow with feathers as its primary source of carbon, sulfur, and energy. Subsequently, a feather-hydrolytic, endospore-forming, motile, rod-shaped bacterium was isolated from the feather-degrading culture. The organism was Gram stain variable and catalase positive and demonstrated facultative growth at thermophilic temperatures. The optimum rate of growth in nutrient broth occurred at 45 to 50°C and at pH 7.5. Electron microscopy of the isolate showed internal crystals. The microorganism was identified as *Bacillus licheniformis* PWD-1. Growth on hammer-milled-feather medium of various substrate concentrations was determined by plate colony count. Maximum growth (approximately 10^9 cells per ml) at 50°C occurred 5 days postinoculation on 1% feather substrate. Feather hydrolysis was evidenced as free amino acids produced in the medium. The most efficient conditions for feather fermentation occurred during the incubation of 1 part feathers to 2 parts *B. licheniformis* PWD-1 culture (10^7 cells per ml) for 6 days at 50°C. These data indicate a potential biotechnique for degradation and utilization of feather keratin.

Feather waste, generated in large quantities as a by-product of commercial poultry processing, is nearly pure keratin protein (7). Keratin in its native state is not degradable by common proteolytic enzymes such as trypsin, pepsin, and papain. However, keratin does not accumulate in nature, and keratinolytic activity has been reported for species of *Aspergillus* (W. Koh, A. Santro, and R. Messing, Bacteriol. Proc., p. 18, 1958), *Ctenomyces* (14), and *Streptomyces* (9). Molyneaux reported the digestion of wool keratin by a *Bacillus* species (6).

Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Prior to being used, the feather is steam pressure-cooked or chemically treated to make it more digestible. These treatment processes require significant energy and also destroy certain amino acids (10–12, 17). Biodegradation by microorganisms possessing keratinolytic activity represents an alternative method to improve the nutritional value of feather waste. Only limited information regarding such microbial treatment of feathers is currently available (5, 8).

In this laboratory, a thermophilic poultry waste digestor system for the treatment of poultry waste and the production of biogas has been developed (15). A bacterial culture that grows on feathers as the sole organic substrate for supplying carbon, sulfur, and energy was enriched from a laboratory poultry waste digestor (18). This study describes the identification and characterization of an isolate from this culture. Additionally, a comparative study of feather fermentation conditions for the production of free amino acids by the microorganism is described (U.S. patent 4.908.220, March 1990).

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† Paper no. 12402 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.

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MATERIALS AND METHODS

Media and culture conditions. The basal medium used for isolation, maintenance, growth, and fermentation analysis of the feather-degrading microorganism contained the following (in grams per liter): NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl \cdot 6H_2O$, 0.1; yeast extract, 0.1; agar, 20 (as needed). pH was adjusted to 7.5. Feathers (ball milled, hammer milled, or whole) were added to the basal medium in various amounts, as will be described below. Feathers were washed in distilled water prior to being sterilized by autoclaving (120°C at 15 lb/in^2 for 20 min). In some stages of the study, feathers were autoclaved for less than 20 min, and sterilization was completed by fumigation with formaldehyde gas (3).

Nutrient broth, nutrient agar, and yeast extract were from BBL Microbiology Systems (Cockeysville, Md.); other media used in the various tests for identification and characterization of the microorganisms were from Difco Laboratories (Detroit, Mich.). For growth determination, the microorganisms were cultured in 250-ml Erlenmeyer flasks containing 50 ml of 1% hammer-milled-feather medium or nutrient broth. Growth at 25, 37, and 50°C with aeration by rotation of the flasks at 175 rpm was determined by plate count on nutrient agar. To ensure an adequate distribution of the cells when plating them on nutrient agar, several 1-mm glass beads were included in the first dilution tube (K. L. Bott, personal communication).

Light microscopy. Standard microbiological methods were used to fix the cells to slides for Gram staining and observation. Endospore staining was by the method of Dorner (13).

Electron microscopy. A pellet of stationary-phase cells from nutrient broth was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% OsO_4, dehydrated through a graded series of ethanol washes, and embedded in an epoxy resin. Uranyl acetate-lead citrate-stained thin sections were viewed with a transmission electron microscope.

Adaptation of bacterium for maximum feather degradation. Whole intact feathers were autoclaved for various periods (1 to 20 min). Single feathers were placed in test tubes contain-

ing 10 ml of basal salt solution and inoculated with the pure, feather-degrading culture. Seven to ten days after inoculation at 50°C, bacteria from the tube showing the most feather degradation were aseptically transferred to fresh whole-feather medium for further selection. After several months of this protocol, a strain able to degrade feathers that had been steam treated for a minimum of 2 min was selected for further study.

Determination of feather degradation under aerobic and anaerobic growth conditions. Feather-degrading cells were grown in 500 ml of 1% hammer-milled-feather medium for 3 days, filtered through four layers of sterile cheesecloth, and collected and washed three times in the basal salt solution. These cells were suspended in the basal salt solution and transferred to culture tubes containing 10 ml of 1% hammer-milled-feather medium. These tubes were maintained under both aerobic and anaerobic conditions (anaerobic tubes were bubbled with O_2-free N_2 for 30 min prior to cell transfer, with the headspaces flushed with the same both during transfer and after transfer until sealed by rubber septa and screw caps). All tubes were incubated at 50°C for 10 days (shaking at 125 rpm). The cultures were sampled daily for cell numbers by determining CFU on ball-milled-feather agar plates, and soluble sulfhydryl concentrations were determined by the method of Ellman (4), as modified by Shih et al. (16).

Solid-phase fermentation of feathers. The feather-degrading microorganism was grown aerobically to a concentration of approximately 10^7 cells per ml in the basal medium containing 1% hammer-milled feathers. This liquid culture was aseptically added to 200-ml plastic bottles containing 5 g of sterile hammer-milled feathers in order to obtain ratios of 1 part feathers (dry weight) to 2, 4, and 8 parts liquid cell culture. The bottles were flushed with oxygen-free nitrogen, sealed with screw caps, and incubated at 50°C. Agitation was by vigorous manual shaking twice per day. Duplicate samples of the aqueous fermentation products were collected on days 0, 3, 6, 12, and 24 after incubation. The liquid was collected by filtration through four layers of cheesecloth to remove nondegraded feather particles. This filtrate was passed through a 0.45-μm-pore-size filter and frozen until amino acid determination.

Amino acid analysis. Samples (200 μl) of the filtrate noted above were methanol extracted (800 μl of 40% methanol in 0.1 N HCl) and centrifuged at 13,000 × *g* for 10 min at 4°C. The supernatant was analyzed for free amino acids by high-performance liquid chromatography. The analysis was performed at the North Carolina State University Protein Sequencing Facility.

RESULTS

Isolation and adaptation of feather-degrading microorganism. It was found that a previously enriched, feather-degrading culture (18) contained microorganisms exhibiting at least two distinct colony morphologies when streaked onto nutrient agar plates. One type contained only rod-shaped bacteria; the other was a coccus, which appeared singly and in chains. Although each type displayed clearing zones when streaked onto the ball-milled-feather agar plates, the rod-shaped bacterium demonstrated the most pronounced clearing zones. This isolate was selected for the purpose of identification and for its adaptation to feather degradation. Cells of the isolate were grown on whole feathers and transferred at frequent intervals to the basal medium, containing whole feathers, in which the autoclave treatment time was systematically reduced. Eventually, after several months of repeated selection, the isolate was able to degrade feathers which had been autoclaved for only 2 min. Attempts to maintain the growth of the isolate on non-steam-treated feathers were not successful.

Identification and characterization of feather-degrading isolate. Microscopic observation of the isolate showed a straight rod (2 to 3 by 0.5 to 1 μm) (Fig. 1) with endospores (Fig. 2). The bacterium grew aerobically, was strongly catalase positive, and was Gram stain variable. Transmission electron micrographs showed, in some cells, internal crystals in a rectangular array with regular spacing (Fig. 1B). Collectively, these characteristics indicated that the isolate was of the genus *Bacillus*.

The optimum rate of growth in hammer-milled-feather medium occurred at temperatures near 50°C (Fig. 3). No growth occurred at temperatures in excess of 65°C. Additional morphological and physiological tests conducted on the isolate in this laboratory were confirmed by the American Type Culture Collection, which characterized the isolate as thermophilic *Bacillus licheniformis*. We propose PWD-1 as the strain designation to indicate isolation of the strain from a poultry waste digestor.

Degradation of feathers by isolate. It was observed that aerobic growth by the isolate on feathers, with the feathers as its primary source of carbon, energy, and sulfur, resulted in nearly complete degradation of the keratin protein after 7 to 10 days of incubation at 50°C. Washed cells from such aerobically grown cultures were inoculated onto fresh hammer-milled-feather medium and incubated at 50°C under both aerobic and anaerobic conditions. Biodegradation was measured as the appearance of soluble sulfhydryl in the growth medium. Growth of the isolate and the concentrations of soluble sulfhydryl under these conditions are shown in Fig. 4. The amount of soluble sulfhydryl present was proportional to the number of viable cells. These results are similar to those previously observed for the enriched, feather-degrading culture (15), although the concentration of soluble sulfhydryl is higher for the pure culture isolate. On the basis of (i) previous evidence that free amino acid concentrations were higher for the enriched culture under anaerobic conditions and (ii) data showing that neither the enriched culture nor pure isolate actively grows anaerobically (Fig. 4), the effect of anaerobic incubation time and the ratio of feather substrate to liquid cell culture on amino acid liberation was examined in greater detail. The results are shown in Fig. 5, and the profiles of the specific amino acids that were liberated are shown in Table 1. The profiles of the amino acids that were measured were relatively similar for all conditions of fermentation. Glutamic acid, alanine, and the branched-chain amino acids isoleucine, leucine, and valine were consistently the most abundant amino acids. The mole percent of arginine decreased significantly under anaerobic conditions.

DISCUSSION

A bacterium isolated from a thermophilic poultry waste digestor has been shown to degrade feather keratin by using feathers as a primary source of energy, carbon, and sulfur. The isolate, which grows optimally at thermophilic temperatures, has been classified as *B. licheniformis* PWD-1. The bacterium was isolated from an anaerobic habitat; however, it showed maximum growth under aerobic conditions, as would be expected of a member of the family *Bacillaceae*. One explanation for the presence of this species in a poultry

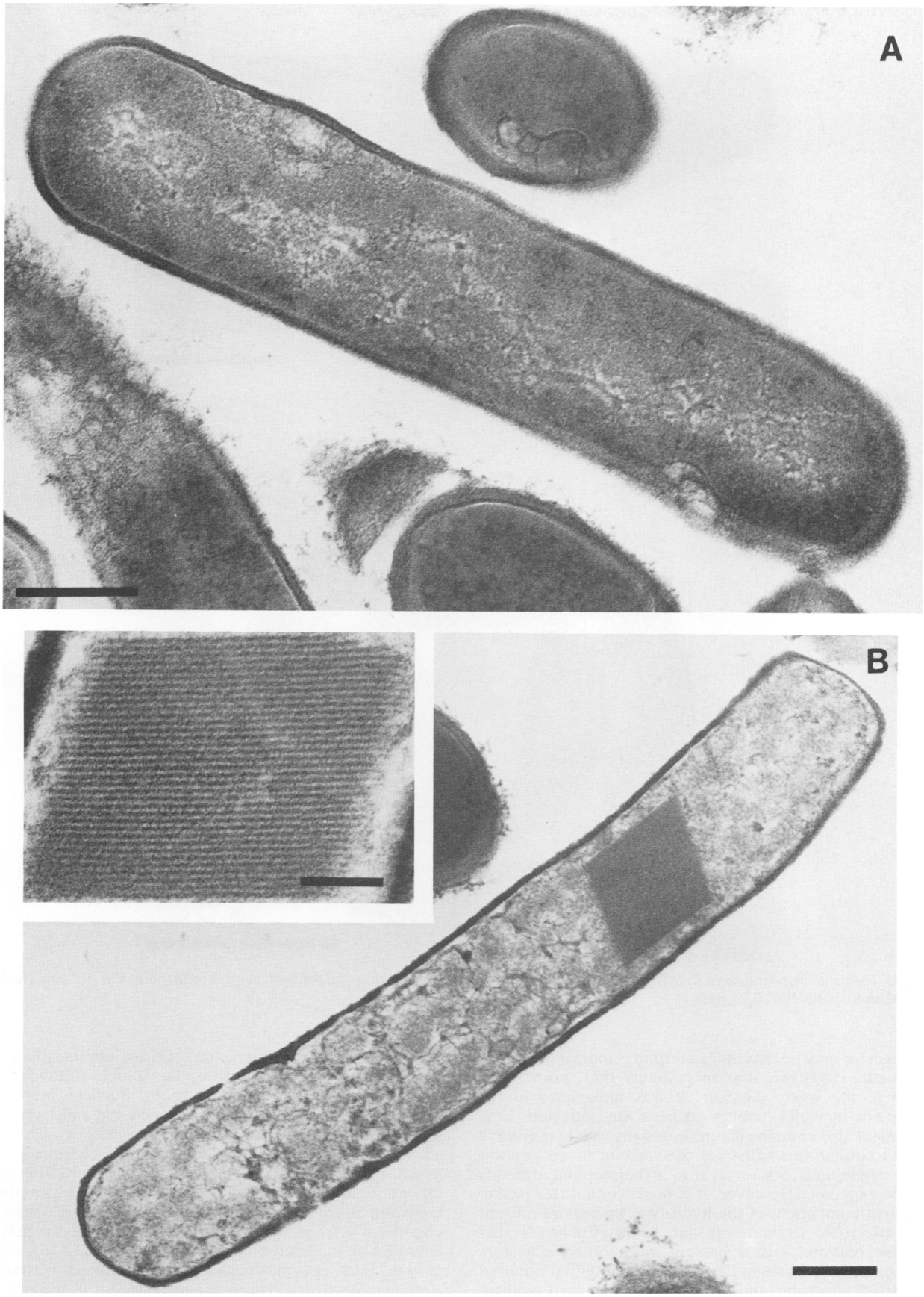

FIG. 1. Electron micrograph of *B. licheniformis* PWD-1. Longitudinal sections of a vegetative cell (A) and of a cell containing a crystal inclusion (B) are shown. Bar, 0.25 μm. Insert: Section of a *B. licheniformis* PWD-1 crystal. Bar, 0.1 μm.

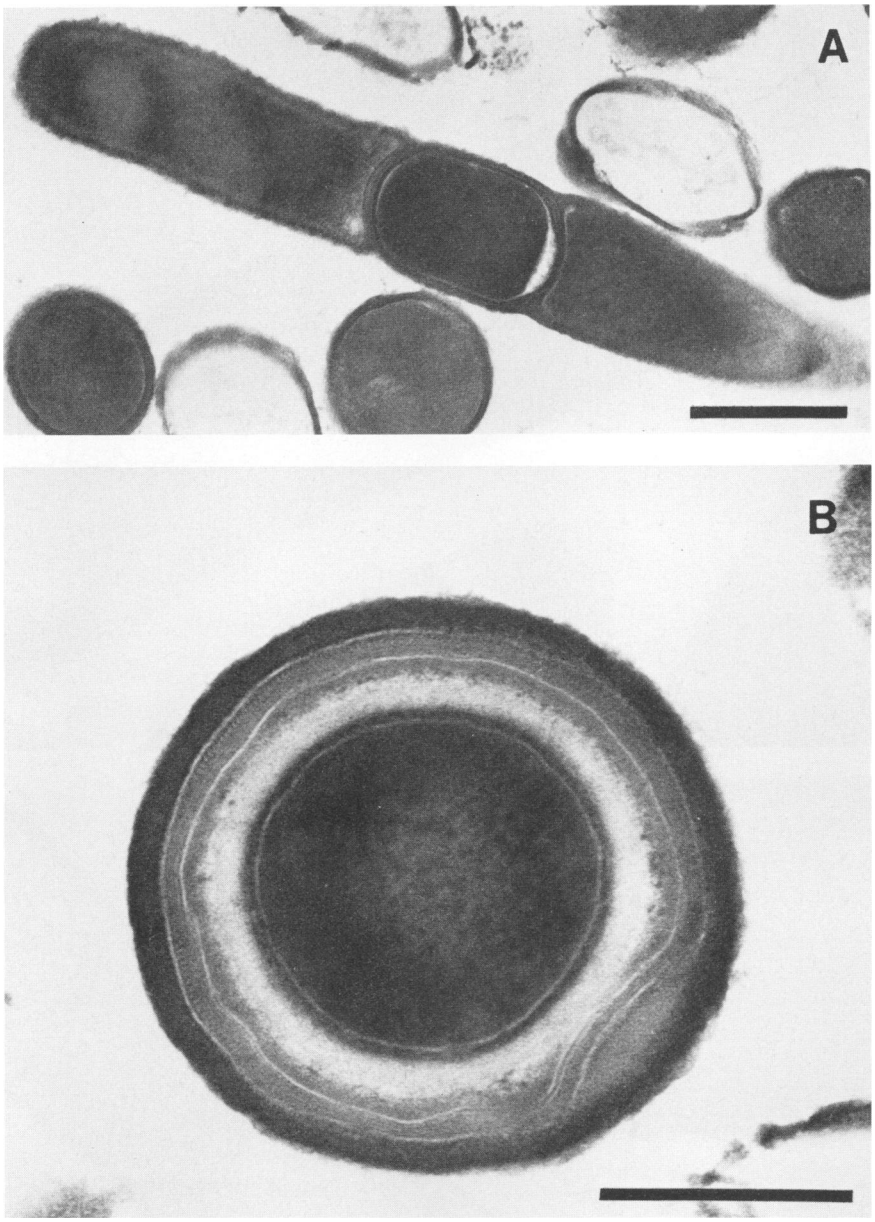

FIG. 2. Electron micrographs of *B. licheniformis* PWD-1. (A) Longitudinal section of a cell with central endospore. Bar, 0.5 μm. (B) Cross section of endospore. Bar, 0.25 μm.

waste digestor may be that the bacterium is indigenous to the chicken gut. However, it is more likely that, prior to its addition to the waste digestor, it was indigenous to the environment in which poultry excreta are collected. This environment also contains feathers, and the isolate may have adapted to utilize this substrate. Because of its endospore-forming capabilities, its survival in a thermophilic digestor would be expected. However, it is possible that this isolate is an active component of the hydrolytic microflora present in the anaerobic digestor. It has been established that facultative microorganisms present in anaerobic digestors are useful in reestablishing the low redox potential required by the strict anaerobes during times when oxygen is introduced with the substrate (2).

When cells of *B. licheniformis* PWD-1 were grown aerobically on feather medium, they yielded appreciable degradation products as measured by the soluble sulfhydryl concentration in the fermentation broth. Such an increase in soluble sulfhydryl did not occur when the cells were subjected to anaerobic growth conditions (Fig. 4). This is in accord with an earlier study in which these parameters were measured in the feather-degrading mixed culture from which this bacterium was isolated. It is noteworthy that the increase in sulfhydryl concentration initially lags behind the growth of the bacterium. This may be due to enzymes liberated by the bacterium following a period of logarithmic growth. Such enzymes have been documented in strains of *B. licheniformis* (1). These enzymes appear in the extracellular medium only during the postlogarithmic phase of growth. The increase of soluble sulfhydryl compounds dur-

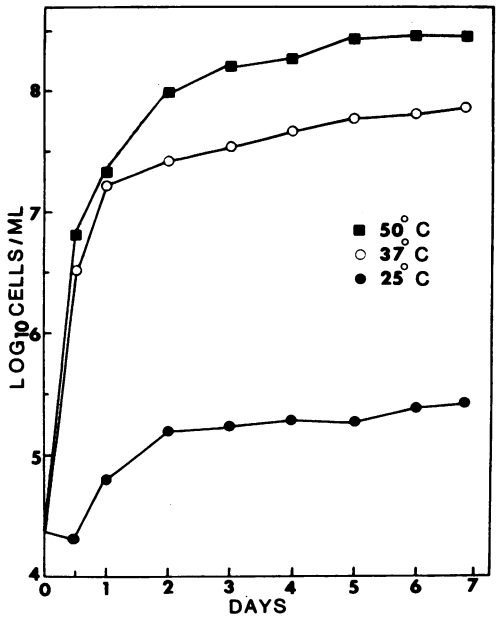

FIG. 3. Growth of *B. licheniformis* PWD-1 at different temperatures in feather medium.

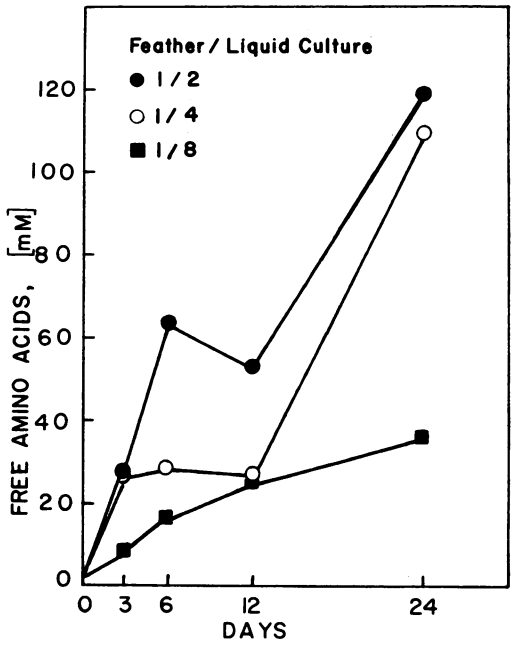

FIG. 5. Total free-amino-acid concentrations in the feather fermentation medium at different ratios of feathers and liquid culture.

ing the degradation of feathers by this strain of *B. licheniformis* is evidence that this bacterium possesses a protease(s) capable of reducing the disulfide bonds of keratin.

Although there was not much growth of the bacterium during anaerobic incubation (Fig. 4), the amount of free amino acids accumulated in the medium was greater during anaerobic incubation than during aerobic growth. It is possible that rapid catabolism of the newly hydrolyzed amino acids occurred during aerobic growth. We used a broth culture containing the bacterium, which had been grown aerobically on a feather medium to approximately 10^7 CFU/ml, to inoculate fresh hammer-milled feathers under conditions made anaerobic for fermentation. Under these conditions, feather degradation continued, and because there was little or no cell growth, a free product in the form of amino acids accumulated in the medium (Table 1). The most efficient production of free amino acids was with a semisolid fermentation medium of 1 part feathers to 4 parts liquid culture, incubated for 6 days. Under these conditions, total free amino acids in the fermentation liquid were measured at a concentration in excess of 60 mM. Although higher concentrations of free amino acids were measured at day 24, the excess incubation time is not practical, especially considering the energy required for thermophilic incubation. The treatment of feathers by *B. licheniformis* in semisolid fermentation medium seems to be a useful method for generating partially hydrolyzed feathers, which in turn can be used as a digestible feed protein (C. M. Williams, C. G. Lee, J. D. Garlich, and J. C. H. Shih, submitted for publication).

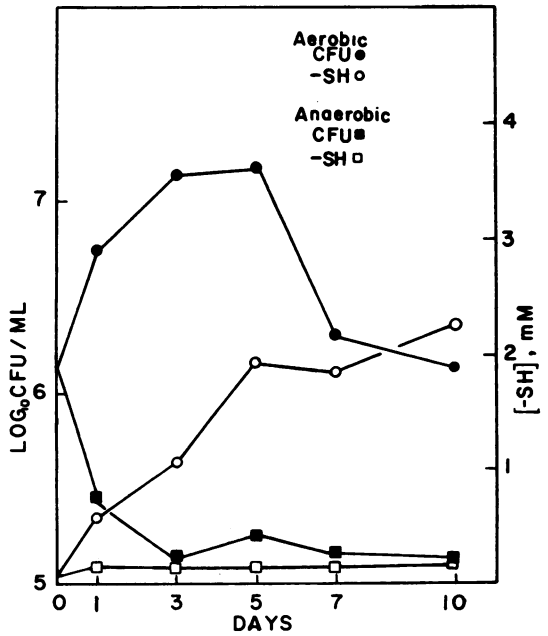

FIG. 4. Growth of *B. licheniformis* PWD-1 and soluble sulfhydryl (-SH) concentrations in the growth medium under aerobic and anaerobic conditions at 50°C.

The degradation of feathers by a *Bacillus* sp. has not been reported previously; however, in 1959 Molyneaux reported the isolation, from the dermoid cyst of a sheep, of a *Bacillus* sp. which digested wool keratin (6). He noted that although the growth of various bacilli on wool had been previously reported, his isolate was the first that could degrade nonsteam-sterilized or native wool keratin. Molyneaux conducted an extensive classification of his isolate but did not assign a species name. Many of the results of biochemical tests he conducted match those we observed for *B. licheniformis* PWD-1. However, the bacillus isolated by Molyneaux grew better under mesophilic temperatures and was also unable to reduce nitrate to nitrite. Additionally, electron microscopy of PWD-1 showed some unique properties. Many cells contained internal crystals, and some demonstrated multiple septa. On the basis of our knowledge of *Bacillus thuringiensis* and other crystal-forming bacilli, it would be logical to assume that the crystal is protein.

TABLE 1. Free-amino-acid concentrations[a] in fermentation medium as affected by fermentation time and ratios of feather substrate to liquid culture[b]

Amino acid	mol% at various feather/liquid-culture ratios														
	Day 0			Day 3			Day 6			Day 12			Day 24		
	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8	1:2	1:4	1:8
Aspartic acid	3.53	3.12	2.90	3.24	3.12	1.87	4.25	3.36	2.40	3.51	3.05	3.30	3.25	2.98	2.93
Glutamic acid	14.19	13.75	13.41	9.92	8.52	7.10	8.81	8.90	7.47	8.22	7.52	8.46	7.56	8.17	8.46
Cysteic acid	1.63	1.92	0.96	0.81	0.93	0.88	0.55	0.90	0.41	0.61	0.44	0.39	0.44	0.41	0.47
Hydroxyproline[c]	5.49	5.19	9.50	1.20	0.69	1.87	0.51	0.65	0.95	0.53	0.42	0.63	0.34	0.32	0.43
Serine	3.08	2.66	2.44	1.06	1.04	1.68	0.49	0.29	1.81	0.35	0.13	2.09	0.51	0.18	0.32
Glycine	8.66	8.64	8.11	4.29	4.27	4.19	6.41	5.37	4.36	6.65	6.70	5.44	6.03	6.66	5.39
Histidine	5.12	4.08	4.44	2.52	1.32	1.38	1.81	1.50	1.62	2.40	1.66	1.80	2.45	2.44	2.11
Arginine	24.85	24.33	17.48	6.51	4.37	4.93	2.83	4.31	2.91	3.02	2.28	1.61	1.61	1.45	1.90
Threonine	2.40	2.47	2.80	2.56	3.54	3.81	2.02	2.37	2.84	1.53	1.71	2.05	1.20	1.41	2.05
Alanine	8.45	9.09	8.28	11.51	12.46	10.19	12.25	13.38	11.63	11.87	12.89	10.90	11.86	11.74	11.79
Proline	4.20	5.20	3.58	1.90	2.89	1.53	2.74	3.92	1.86	1.81	3.09	1.75	4.33	5.92	4.39
Tyrosine	1.80	2.03	2.90	2.85	3.50	4.43	3.29	3.67	4.65	3.89	4.21	5.02	3.42	3.19	3.38
Valine	3.48	3.28	2.83	9.23	9.94	9.57	10.33	9.03	10.95	11.17	11.01	11.07	9.65	9.62	10.44
Methionine	0.79	0.60	4.87	2.52	3.42	2.94	3.31	3.05	3.46	3.28	3.42	3.86	3.83	3.51	3.66
Cysteine	0	0	0	0.32	0	0	0	0	0	0.26	0	0	0	0	0
Isoleucine	1.48	1.66	2.07	6.92	8.58	7.75	8.24	8.44	8.90	8.54	9.59	9.26	9.17	9.41	8.89
Leucine	3.06	3.45	3.76	14.13	13.91	14.77	13.18	13.43	15.37	14.32	15.52	14.45	13.16	13.47	14.66
Phenylalanine	1.76	2.10	2.10	5.60	6.74	7.28	6.88	6.78	7.37	6.27	6.85	7.55	5.79	5.65	6.07
Tryptophan	1.16	1.06	1.70	7.45	6.53	6.62	7.80	6.72	6.72	7.39	5.68	6.58	6.27	6.63	8.47
Lysine	4.88	5.37	5.87	5.46	4.25	7.20	4.29	3.91	4.34	4.39	3.81	3.80	9.02	6.85	4.20
Total (mM)	3.95	2.80	1.05	25.20	23.30	7.60	63.20	26.45	15.60	52.80	25.25	26.70	118.00	110.25	36.90

[a] Values represent duplicate-sample means.
[b] Liquid culture of *B. licheniformis* PWD-1 contained approximately 10^7 cells per ml.
[c] Values include a 200-pmol hydroxyproline spike for chromatographic analysis. This value is taken into account for total millimolar concentration.

However, the origin, composition, and significance of this ultrastructure in *B. licheniformis* is not known.

Extensive efforts were made to adapt PWD-1 to degrade native feather keratin, but attempts to maintain a viable culture which could consistently degrade non-steam-treated feathers were unsuccessful. A minimum autoclave time of 2 min was needed for repeated feather degradation activity. Apparently, some steam treatment is required to render native feather keratin accessible for hydrolysis by this bacterium. We have recently conducted studies which indicate that the application of this bacterium to feathers subjected to a brief steam treatment followed by controlled biodegradation is energy efficient and produces a digestible protein (Williams et al., submitted). These data indicate a potential new biotechnique for the degradation and utilization of feather keratin.

ACKNOWLEDGMENT

Grant support from the Southeastern Poultry and Egg Association is appreciated.

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